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Original Article

Effects of transportation time after extraction on the magnetic cryopreservation of pulp cells of rat dental pulp

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KEYWORDS
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Abstract *Background/purpose:* The aim of this study was to examine the effects of the time period between tooth extraction and the freezing procedure on pulp cells in frozen intact teeth of rats.
Material and methods: In total, 120 incisors from 30 rats were extracted and kept in transportation solution for 0 hour, 12 hours, and 24 hours. The tested teeth were divided into 2 experimental groups, where the extracted incisors were first frozen in a magnetic-programmed freezer (PF) or a traditional −20°C freezer (TF). The tested teeth were then stored at −150°C for 7 days. Incisors extracted from the opposite side of the same rat were treated as the non-frozen control. After thawing, the pulp was extracted and dissected. Hematoxylin and eosin staining was performed to observe the cell distributions. Cell densities in the odontoblast region and cell-rich zone were calculated using an optical microscope.

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Results: When samples were immediately frozen after extraction, the cell density of odontoblastic cells was higher in the PF group compared with analogs which were frozen in the −20°C freezer ($P < 0.05$). In the cell-rich zone, cell densities of the PF group were significantly higher ($P < 0.05$) than those of the TF group although the pulp cells had been stored in transportation solution for 24 hours before being frozen.
Conclusions: The freezing technique used in this animal study provided positive effects on pulp cell storage. In addition, the storage time before the freezing procedure is an important issue for cryopreserving pulp cells in intact teeth.

Introduction

Regeneration is the mainstream in medical science in the 21st century, and also a goal for which scientists keep searching. The acquisition of good sources of stem cells (SCs) and accurately induced differentiation ability are 2 major factors for the success of regenerative medicine, and are also research subjects that scientists continue exploring. Meanwhile, determining ways to preserve the source tissue intact for further SC extraction is an important cornerstone of the development of SC technology.[1,2]

Since Gronthos et al. first discovered SCs in permanent teeth, diverse studies of pulp SCs have gradually become the focus of tissue engineering.[3,4] However, if mesenchymal SCs are deliberately extracted but not used in a timely manner, the entire process can be a waste of time and money. In this regard, an alternative way for dental tissue engineering is to save both the periodontal ligaments and pulp tissues at the same time, and thaw them for teeth reimplantation or mesenchymal SC extraction if needed in the future.

For cryopreservation of dental tissues, almost all studies focused on the cryopreservation of periodontal ligaments. Kaku and colleagues reported that the survival rate of reimplanted pulpless teeth after thawing was up to 97%.[5] This result demonstrates the possibility of the clinical application of a tooth bank.[5] In 2001, Laureys et al. froze pulpless teeth for 7 days and found that revascularization could be activated when the teeth were thawed and reimplanted.[6] However, technical developments concerning the cryopreservation of intact teeth with pulp are still limited. To cryopreserve pulp tissue, Lee and colleagues,[7] using the same technique as Kaku et al.[5] to cryopreserve extracted intact teeth, found that after thawing, dental pulp SCs could be successfully incubated from 73% of the thawed teeth. This result suggested that cryopreserved intact teeth not only can be used for autotransplantation but also can provide a source of dental pulp SCs.[7]

However, whether the storage time between teeth extraction and the freezing procedure influence the preserved cells is still unknown. Thus, in this animal study, we investigated the influence of storage time before cryopreservation on pulp cells thawed from cryopreserved intact teeth of the rat.

Materials and methods

Thirty Wistar rats (with an average weight of 91.4 ± 19.4 g) used in this study were purchased from the National Laboratory Animal Center (Taipei, Taiwan). Central incisors of the maxilla and mandible of the 30 rats were extracted before the experiments. To obtain intact incisors, a rat was sacrificed, and its jawbone was excised. Then the surrounding bone of the incisor was carefully removed. Immediately after extraction, the teeth were cleaned with phosphate-buffered saline (PBS) and stored in a normal saline solution. Thirty extracted teeth were frozen in a programmed freezer (ABI, Chiba, Japan), supplied with a magnetic field (PF group), as described in a previous report.[7] Briefly, the teeth were transported at 4°C and then placed in a freezer at −5°C. Teeth were maintained at that temperature for 15 min, and then cooled at a rate of −0.5°C/min until the temperature reached −32°C. During the freezing process, an electromagnetic field from a 75-mA current passed through a coil was applied to the samples. For the traditional freezing group (TF group), 30 extracted teeth were frozen in a traditional −20°C freezer. During cryopreservation, the teeth were immersed in commercialized dimethyl sulfoxide (DMSO)-free cryoprotectant (modified-BAMBANKER, Lymphotec, Tokyo, Japan). After the freezing procedure, all experimental teeth were transferred to a freezer (MDF-11561, Sanyo, Osaka, Japan) and stored at −152°C for 7 days. For each group, incisors extracted from the opposite side of the same rat were treated as the control. These control teeth were not subjected to either the freezing procedure or 7-day cryopreservation. To test the effects of transportation time between teeth extraction and the freezing procedure on pulp cells, the extracted incisors in the 2 experimental groups were kept in transportation solution for 0 hour, 12 hours, and 24 hours, with 10 samples used for each experimental condition.

After thawing at room temperature, teeth samples were fixed in 10% formalin and a decalcification solution in 10% formic acid for 48 hours according to a previous report.[8] Then a tooth sample was horizontally cut into cubes and dehydrated using a graded series of alcohol. After dehydration, the tooth cubes were embedded in paraffin and dissected at 6 μm with a sliding microtome (Leica LM2500, courtesy of Meyer Instruments, Langham Creek, Houston, TX, USA). The slices were then dewaxed, stained with hematoxylin and eosin (H&E), and examined with an optical microscope.

Four microscopic images were taken of each pulp slice. For each microscopic image, two 40 × 15-μm squares were respectively defined at the odontoblast region and cell-rich zone (Fig. 1). Cell numbers were calculated in each square. Quantitative analysis was performed by comparing cell

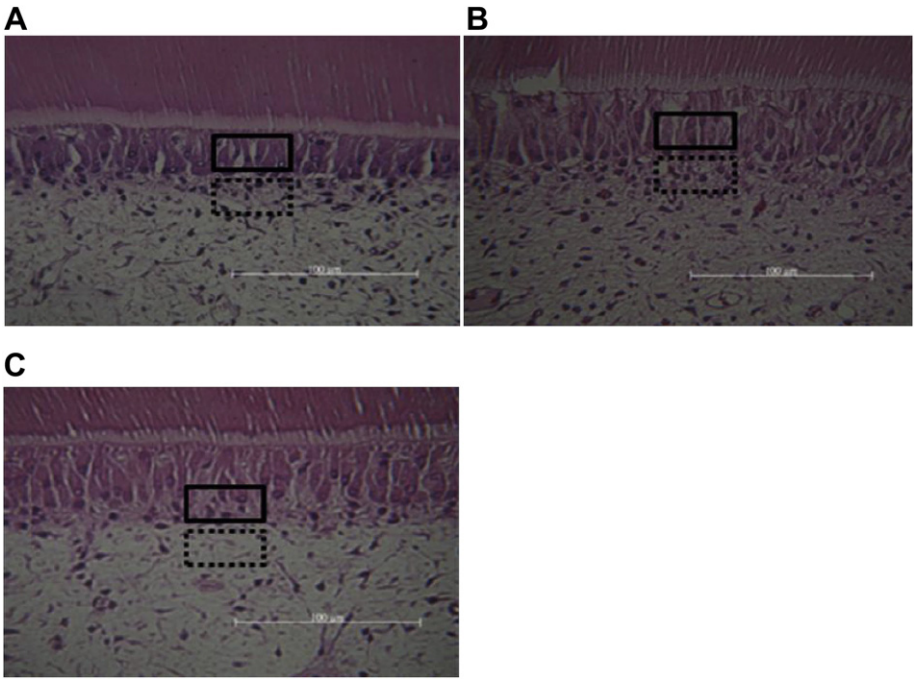

Figure 1 Microscopic images demonstrating a typical slice from the pulp of a Wistar rat tooth. After 24 hr of storage in transportation solution, samples were cryopreserved using (A) a magnetic-programmed freezer and (B) a traditional −20°C freezer. The non-frozen group was used as a control group (C). The solid and dotted frames respectively indicate the location of the odontoblastic region and cell-rich zone.

densities obtained under the various experimental conditions. Analysis of variance with Tukey's post-hoc test was used for the statistical analysis.

Results

To examine the effect of storage time before freezing on dental pulp cells subjected to magnetic freezing, −20°C freezing, and non-freezing, thawed samples were fixed, sliced, and stained with H&E. For the 0-hour transportation time, cell densities of odontoblasts in the PF, TF, and non-frozen control groups were respectively 93.1 ± 16.0, 74.5 ± 7.9, and 130.4 ± 6.8 cells/mm^2. Statistical analysis showed that there were significant differences among the groups ($P < 0.05$) (Fig. 2A). Cell densities in the cell-rich zone were 74.7 ± 18.8, 63.7 ± 16.0, and 95.2 ± 16.4 cells/mm^2 for the PF, TF, and, non-frozen control groups, respectively (Fig. 2B). For both regions analyzed, the statistical analysis demonstrated that cell densities for the PF group were significantly higher than analogous values of the TF group ($P < 0.05$).

For samples stored in transportation solution for 12 hours before undergoing the freezing procedure, cell densities of the 2 frozen groups, in both the odontoblast region (Fig. 3A) and cell-rich zone (Fig. 3B), were significantly lower than those of the non-frozen samples ($P < 0.05$). A significant difference between the PF (54.8 ± 15.9 cells/mm^2) and TF groups (47.4 ± 13.0 cells/mm^2) was only found in the cell-rich zone (Fig. 3B) ($P < 0.05$). When the extracted teeth were cryopreserved after 24 hours of storage in transportation solution, similar results were obtained (Fig. 4). A significant difference between the PF (52.3 ± 19.4 cells/mm^2) and TF groups (39.9 ± 12.0 cells/mm^2) was only found in the cell-rich zone (Fig. 4B) ($P < 0.05$).

Discussion

In this study, we tested the effects of storage time between tooth extraction and the freezing procedure on pulp cells in a frozen intact tooth. Our data showed that anatomical patterns and cell distributions in each layer of pulp tissues using different freezing methods could clearly be distinguished. In addition, no difference in the cell morphology was found when the frozen and non-frozen groups were compared (Fig. 1). Nevertheless, data in Figs. 2–4 demonstrate that when the freezing procedures were performed immediately after the tooth was extracted, the maximum number of pulp cells from thawed intact teeth was obtained. In addition, when the storage time increased, the numbers of preserved cells gradually decreased. Interestingly, from Figs. 3 and 4, we found that pulp cells in the cell-rich zone could withstand the magnetic freezing procedure better than the traditional −20°C freezer, even when the tested teeth were pre-stored in transportation solution for 24 hours.

It is well known that the most essential condition of successful freezing is to avoid the formation of ice crystals. From the results of this study, the magnetic-programmed freezer can produce better cryopreservation effects than a traditional −20°C freezer. The reason could be the AC magnetic field generated by the 75-mA current added to the process of freezing. This generates an electron

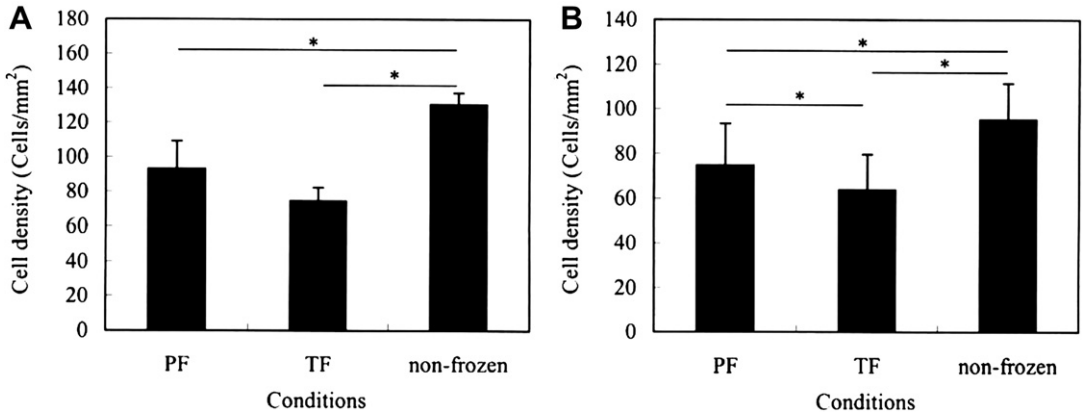

Figure 2 Cell densities in the programmed freezer (PF), −20°C freezer, and non-frozen groups. Test samples were stored in transportation solution for 0 hr before the freezing procedure. (A) and (B) are data from the odontoblastic region and cell-rich zone, respectively. Student's *t* test was used as the statistical method. * Indicates a significant difference ($P < 0.05$).

paramagnetic resonance effect in the bonding electrons of water molecules, so that the bonding kinetic energy is not zero, enabling the water molecules to be instantly frozen with no time to form ice crystals. The vitrification generated by instant freezing can prevent cells from damage caused by ice crystals. Hence, this magnetic freezing technique can efficiently freeze both teeth and pulp cells, which is probably because of the freezing method of vitrification instead of the formation of ice crystals.

The limitation of this study is that no specific staining or distinguishing morphological evaluation were performed to

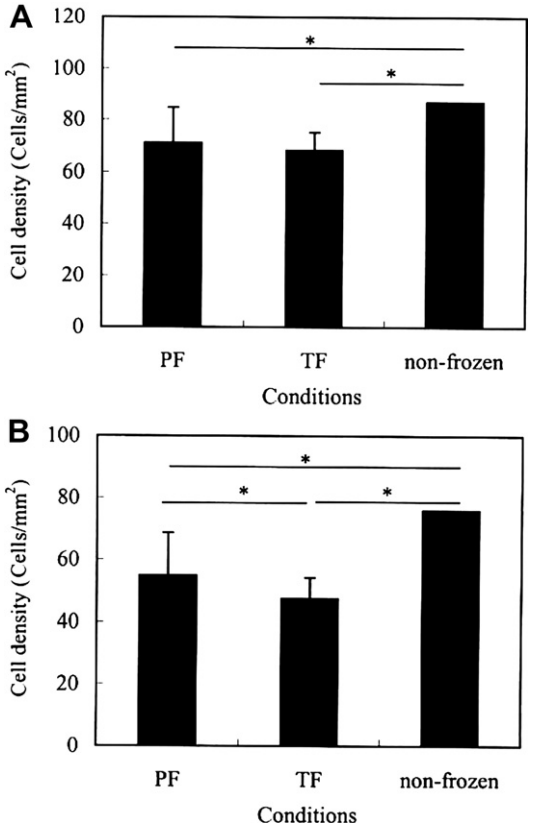

Figure 3 Cell densities in the programmed freezer (PF), −20°C freezer, and non-frozen groups. Test samples were stored in transportation solution for 12 hr before the freezing procedure. (A) and (B) are data from the odontoblastic region and cell-rich zone, respectively. Student's *t* test was used as the statistical method. * Indicates a significant difference ($P < 0.05$).

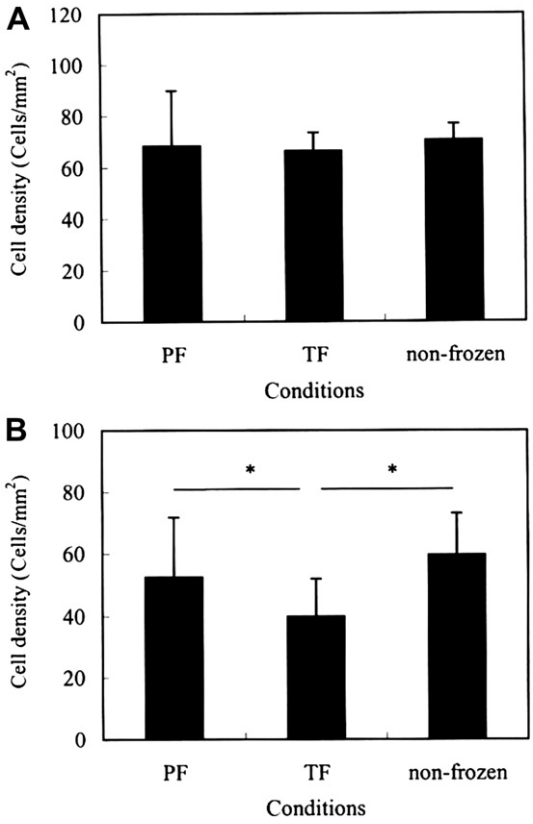

Figure 4 Cell densities in the programmed freezer (PF), −20°C freezer, and non-frozen groups. Test samples were stored in transportation solution for 24 hr before the freezing procedure. (A) and (B) are data from the odontoblastic region and cell-rich zone, respectively. Student's *t* test was used as the statistical method. * Indicates a significant difference ($P < 0.05$).

prove that dental pulp SCs are located in areas where the histological images were examined. However, saving more pulp cells during the cryopreservation procedure of intact teeth still has scientific and clinical meaning for tissue engineering. Since it was proven in the literature that dental pulp cells in pulp tissues still have the ability to recover after thawing,[7] the results of this study will benefit basic knowledge of the preservation of pulp cells. Accordingly, the results of this study demonstrate that the magnetic freezing technique has potential to preserve an entire tooth and achieve the goal of effective pulp preservation without the need to extract pulp cells before cryopreservation.

Conclusions

The results of this study showed that the storage time between teeth extraction and freezing procedure is an important factor influencing the cryopreservation of pulp cells. In addition, we found that pulp cells in the cell-rich zone have better properties of withstanding the freezing procedure.

Acknowledgments

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